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Pu, Dan
Liang, Hao
Wei, Fang
et al.

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ORIGINAL ARTICLE

Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study

Dan Pu^{1†}, Hao Liang^{1†}, Fang Wei^{2†}, David Akin², Ziding Feng³, QingXiang Yan³, Yin Li⁴, Yan Zhen⁴, Lin Xu⁵, Gaochao Dong⁵, Huajing Wan⁶, Jingsi Dong¹, Xiaoming Qiu¹, Changlong Qin¹, Daxing Zhu¹, Xi Wang¹, Tong Sun¹, Wenbiao Zhang¹, Canjun Li¹, Xiaojun Tang¹, Youlin Qiao⁷, David T.W. Wong² & Qinghua Zhou¹

1 Lung Cancer Center, West China Hospital, Sichuan University, Chengdu, China

2 School of Dentistry, Department of Pathology, University of California, Los Angeles, California, USA

3 Department of Biostatistics, MD Anderson Cancer Center, University of Texas, Houston, Texas, USA

4 Department of Thoracic Surgery, Henan Cancer Hospital, University of Zhengzhou, Zhengzhou, China

5 Department of Thoracic Surgery, Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing, China

6 Laboratory of Lung Development and Disease, West China Second University Hospital, Sichuan University, Chengdu, China

7 Department of Cancer Epidemiology, Cancer Hospital/Institute, Chinese Academy of Medical Sciences, Beijing, China

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Correspondence

Qinghua Zhou, Lung Cancer Center, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China.

Tel: +86 28 86298113

Fax: +86 28 86298139

Email: zhouqh135@163.com

David T.W. Wong, School of Dentistry, Center for Oral/Head & Neck Oncology Research, University of California Los Angeles, 10833 Le Conte Avenue, 73-034 CHS, Los Angeles, CA 90095, USA.

Tel: +1 310 206 3048

Fax: +1 310 825 7609

Email: dtww@ucla.edu

[†]These authors contributed equally to the study.

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Abstract

Background: This article describes a pilot study evaluating a novel liquid biopsy system for non-small cell lung cancer (NSCLC) patients. The electric field-induced release and measurement (EFIRM) method utilizes an electrochemical biosensor for detecting oncogenic mutations in biofluids.

Methods: Saliva and plasma of 17 patients were collected from three cancer centers prior to and after surgical resection. The EFIRM method was then applied to the collected samples to assay for exon 19 deletion and p.L858 mutations. EFIRM results were compared with cobas results of exon 19 deletion and p.L858 mutation detection in cancer tissues.

Results: The EFIRM method was found to detect exon 19 deletion with an area under the curve (AUC) of 1.0 in both saliva and plasma samples in lung cancer patients. For L858R mutation detection, the AUC of saliva was 1.0, while the AUC of plasma was 0.98. Strong correlations were also found between presurgery and post-surgery samples for both saliva (0.86 for exon 19 and 0.98 for L858R) and plasma (0.73 for exon 19 and 0.94 for L858R).

Conclusion: Our study demonstrates the feasibility of utilizing EFIRM to rapidly, non-invasively, and conveniently detect epidermal growth factor receptor mutations in the saliva of patients with NSCLC, with results corresponding perfectly with the results of cobas tissue genotyping.

Introduction

Lung cancer is the most common cancer and leading cause of cancer death in China and worldwide.^{1–8} Non-small cell lung cancer (NSCLC) constitutes 80% of all lung cancer cases, and is typically diagnosed at an advanced stage when

survival rates are low.^{9–15} The discovery of a relationship between epidermal growth factor receptor (EGFR)-activating mutations and EGFR-tyrosine kinase inhibitors (TKI) has taken the treatment of patients with EGFR mutations into an era of precision medicine.^{16–29} The 2009

Iressa Pan-Asia Study (IPASS) demonstrated that EGFR-TKIs are superior to traditional chemotherapeutic agents for patients with EGFR mutant lung cancer, and the current clinical EGFR-TKIs have become a standard first-line treatment for patients with non-squamous NSCLC carrying EGFR mutations.²⁰ Genotyping for EGFR mutations in advanced non-squamous NSCLC patients is highly recommended in current clinical practice.

Epidermal growth factor receptor testing for mutations is traditionally performed on tissues acquired by surgery or biopsy. However, the majority of these diagnoses are on late stage patients in poor physical condition. In these instances, the performance of biopsy or surgery is often impractical because of poor patient health and the risk of additional clinical complications. Moreover, a high proportion of NSCLC patients will develop EGFR-TKI treatment resistance, and performing additional surgeries or biopsies to monitor for an additional change in EGFR mutation status would further increase the risk to patient health.^{21–26} As a result of these limitations, explorations have been made into the feasibility of detecting actionable EGFR oncogenic mutations in biofluids, such as serum or plasma.^{3–8}

Recent emerging work from the University of California, Los Angeles (UCLA) has demonstrated the feasibility of performing saliva-based EGFR mutation detection (SABER) based on a core technology called electric field-induced release and measurement (EFIRM).²¹ This method is an electrochemical method based on immobilized nucleic acid probes for capturing mutated sequences and applying electric fields to facilitate the hybridization process. Because of the speed and simplicity of the method, EFIRM has the potential to be a suitable tool for oncogenic mutation monitoring in a clinical context. Recently, a randomized, blind study using SABER for NSCLC patients demonstrated high concordance with tumor tissue based genotyping.²³ Here, we report the first clinical validation pilot study of EFIRM in Mainland China.

Methods

Patients and clinical specimens

Inpatients with suspicious lesions for lung cancer, treated at three large-scale major hospitals in China, West China Hospital, Henan Cancer Hospital, and Jiangsu Cancer Hospital, were recruited as subjects for our study. Each study participant had 5 mL saliva and 10 mL blood collected twice on the day before surgery, the day after surgery, and approximately seven days after surgery. Blood plasma samples were collected by drawing blood into ethylenediamine-tetraacetic acid tubes, centrifuging at 2500g for 10 minutes at -4°C , and then collecting the upper plasma layer. Samples were stored at -80°C until analysis. As per previous

studies in salivary biomarkers, saliva samples were kept on ice during collection from patients and then centrifuged at 2600g for 15 minutes at 4°C .²² The supernatant was removed from the pellet, treated with RNase inhibitor (Superase-In, Ambion Inc., Austin, TX, USA), and stored at -80°C for future analyses. All subjects provided informed consent.

Saliva-based epidermal growth factor receptor (EGFR) mutation detection in bodily fluids

Epidermal growth factor receptor mutations in serum and saliva were detected by EFIRM in blinded samples by trained laboratory personnel. Each plasma and saliva sample was measured in duplicate. Paired probes (capture and detector; TsingKe, Beijing, China) specific to the two TKI-sensitive mutations were used: for the exon 19 deletion (19 del), a capture probe 5'-TGT TGC TTC CTGAT AGC GAC G-3' and a detector probe 5'-GGA ATT TTA ACT TTC TCA CCT-FITC-3'; for the L858R point mutation, a capture probe: 5'-CAG TTT GGC CCG CCC AAA ATC-3' and detector probe: 5'-TTG ACA TGC TGC GGT GTT TTC A-FITC-3'. The detector probes were labeled with fluorescein isothiocyanate. The EFIRM detection method involves four primary steps.²¹ First, copolymerization of capture probes with pyrrole onto the bare gold electrodes by applying a cyclic square wave electric. Second, hybridization of the samples with detector and capture probes. Third, the combination of anti-fluorescein antibody conjugated to horseradish peroxidase (1:1000 dilution; Roche, Indianapolis, IN, USA). Fourth, chromogenesis by 3,3', 5,5'-tetramethylbenzidine substrate for horseradish peroxidase and measurement of the amperometric signal. The total detection time of the protocol was less than 10 minutes, and the procedure required 20 to 40 μL of the biological sample.

EGFR mutation detection in tumor tissues

Epidermal growth factor receptor mutations in tumor tissues were detected by cobas assay, which is an allele-specific real-time polymerase chain reaction (PCR) system that qualitatively measures the amplification of DNA to detect EGFR gene mutation from DNA derived from freshly frozen tissues. DNA from these tissue specimens was extracted according to the standard procedure delineated in the cobas DNA Sample Preparation Kit (Roche Molecular Systems Inc., Pleasanton, CA, USA).²¹ This processed involved first incubating the sample with protease and buffer ATL at 56°C until dissolution. Buffer AL was then added for an additional 10 minutes at 70°C . Next, DNA samples were collected by the DNeasy Mini spin column (Qiagen, Valencia, CA, USA). After DNA samples were collected, the amount of DNA was analyzed by spectrophotometer and

adjusted to a concentration of 2 ng/ μ L. Finally, the prepared DNA was amplified and detected by cobas 4800 (Roche Molecular Systems Inc.) following the instructions for the cobas EGFR mutation test. The results were automatically reported in cobas 4800 software.

Statistical analysis

Receiver operating characteristic curves (ROC) and the area under the curve (AUC) with 95% confidence intervals (CI) were calculated to access the ability of EFIRM in detecting EGFR mutations. Correlation coefficients were calculated using Pearson parametric methods. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The clinical characteristics of patients

A total of 17 randomized patients with adenocarcinoma with EGFR mutation from three different cancer centers in China (10 from West China Hospital, 4 from Henan Cancer Hospital, and 3 from Jiangsu Cancer Hospital) were enrolled. The clinical characteristics of these patients, including gender, age, smoking status, cancer stage, and pathological type, are presented in Table 1.

Electric field-induced release and measurement detection of EGFR mutations in saliva and plasma

The strength of the amperometric currents detected by EFIRM represented the status of the EGFR mutation. The saliva samples of three patients taken prior to surgery showed significantly higher currents using exon 19 probes, which meant that they were detected to have EGFR exon 19 del mutations (Fig 1a; 85.3 ± 0.3 nA in the exon 19 del group vs. 51.2 ± 5.6 nA in the L858R mutant and 49.7 ± 7.4 nA in the wild-type; $P < 0.01$). Similarly, three patients, whose saliva samples had higher current readouts on EFIRM using the L858R mutant probes, were detected to harbor EGFR L858R mutants (Fig 1b; 114.9 ± 26.5 nA in the L858R mutant group vs. 45.6 ± 0.2 nA in the exon 19 del and 53.6 ± 12.0 nA in the wild-type; $P < 0.01$). This detection included the samples collected both before and after surgery to avoid the possibility of tumor cells or DNA dropping into the circulation system by outside forces, and the results showed high consistency (Figs 1a, 2a and 1b, 2b). ROC analysis indicated AUCs of 1 for the probe carrying the exon 19 del and L858R mutation (Figs 1c, 2c).

Plasma samples were collected before and after surgery by EFIRM, except in one patient who only had samples taken after surgery. The results showed three exon 19 deletions (Fig 3a, 85.6 ± 9.6 nA in the exon 19 del group vs.

Table 1 The clinical characteristics of patients in the study

Patient No.	Gender	Age	Smoking status	Histologic type	Stage†	Cobas	EGFR status			
							EFIRM			
							Saliva		Plasma	
							Before	After	Before	After
1	M	76	N	NSCLC	pT2bN0M0, IIA	No	No	No	No	No
2	M	53	Y	Adeno	pT2aN1M0, IIA	Ex19 del	Ex19 del	Ex19 del	Ex19 del	Ex19 del
3	M	53	Y	Squa	pT2bN0M1a, IV	No	No	No	No	No
4	M	53	Y	Adeno	pT2bN2M0, IIIA	Ex19 del	Ex19 del	Ex19 del	Ex19 del	Ex19 del
5	M	65	Y	Squa	pT3N0M0, IIB	No	No	No	No	No
6	F	72	N	NSCLC	pT3N0M0, IIB	G719X	No	No	No	No
7	M	71	Y	Adeno	PT2aN0M0, IB	L858R	L858R	L858R	L858R	L858R
8	M	46	Y	Adeno	pT3N3M0, IIIB	No	No	No	No	No
9	M	57	Y	Squa	pT4N0M0, IIIA	No	No	No	No	No
10	F	61	N	Adeno	pT2aN2M0, IIIA	Ex19 del	NE	Ex19 del	NE	Ex19 del
11	M	47	Y	Adeno	pT2bN2M0, IIIA	No	No	No	No	No
12	M	62	Y	Squa	pT2aN0M0, IB	No	No	No	No	No
13	F	51	N	Adeno	pT2aN0M0, IIB	L858R	L858R	L858R	L858R	L858R
14	M	71	N	Squa	pT1aN1M0, IIA	No	No	No	No	No
15	M	50	N	Adeno	pT2N2M0, IIIA	No	No	No	L858R	L858R
16	M	60	Y	Squa	pT2N0M0, IB	No	No	No	No	No
17	M	51	N	Adeno	pT2N2M0, IIIA	L858R	L858R	L858R	L858R	L858R

†Stage was based on National Comprehensive Cancer Network guidelines 2014, V3. Adeno, adenocarcinoma; EGFR, epidermal growth factor receptor; EFIRM, electric field-induced release and measurement; F, female; M, male; NE, no samples; NSCLC, non-small cell lung cancer; Squa, squamous lung cancer.

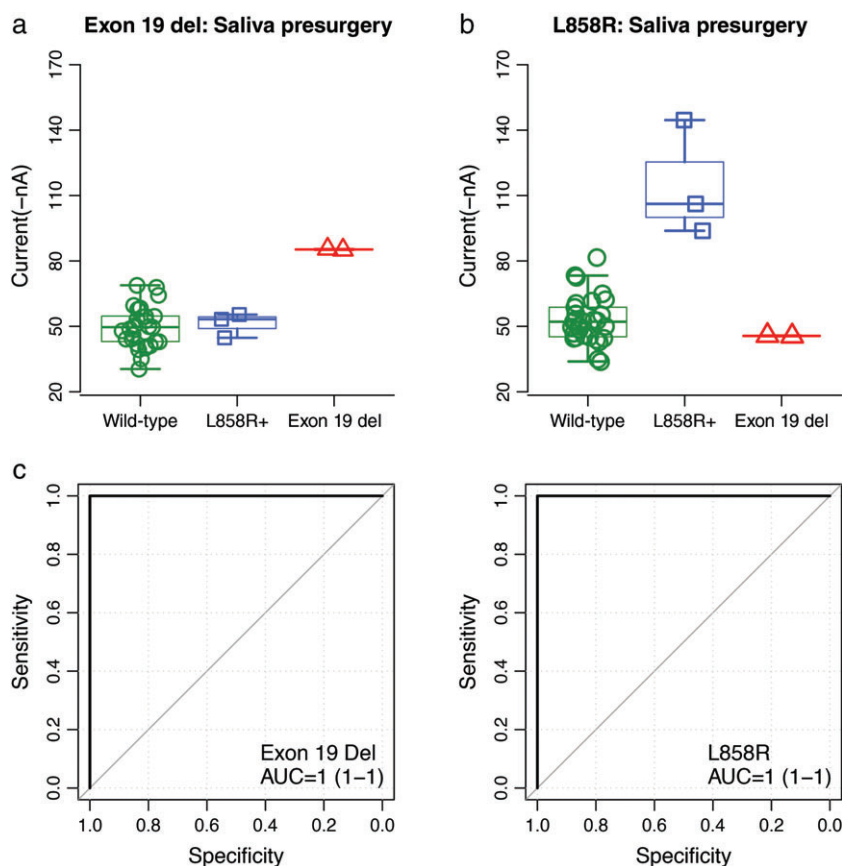


Figure 1 Blinded and randomized detection of epidermal growth factor receptor (EGFR) mutations by electric field-induced release and measurement assay in saliva collected before surgery from patients with non-small cell lung cancer: (a) the probe for EGFR exon 19 deletion in saliva; (b) the probe for L858R in saliva; and (c) receiver operating characteristic curves for detecting EGFR exon 19 deletion (area under the curve [AUC] = 1) and L858R mutations (AUC = 1), respectively.

48.9 ± 8.0 nA in the L858R mutant and 51.1 ± 9.5 nA in the wild-type; $P = 0.001$) and four L858R mutations (Fig 4a, 111.4 ± 27.8 nA in the L858R mutant group vs. 46.2 ± 0.7 nA in the exon 19 del and 51.9 ± 11.8 nA in the wild-type; $P < 0.001$) before surgery. Similar results were obtained from plasma collected after surgery (Fig 3b, 92.6 ± 18.8 nA in the exon 19 del group vs. 52.9 ± 6.1 nA in the L858R mutant and 51.8 ± 5.4 nA in the wild-type; $P < 0.001$; Fig 4b, 108.9 ± 22.2 in the L858R mutant group vs. 45.9 ± 1.7 nA in the exon 19 del and 52.4 ± 13.6 nA in the wild-type; $P < 0.001$). The AUCs are shown in Fig 4c, respectively.

Cobas detection of EGFR mutations in tumor tissues

As a result of the approval of cobas for clinical use by the United States Food and Drug Administration and the China Food and Drug Administration, cobas assay is used as the gold standard method of EGFR mutation detection.

We detected 17 tissue samples and found six samples harboring EGFR mutation (35.3%; 3 exon 19 del and 3 L858R mutations). We compared the results from EFIRM assay using saliva and plasma with the cobas assay using tumor tissues to evaluate the sensitivity and specificity of EFIRM assay in detecting EGFR mutation. The saliva and plasma samples of three patients that were identified to harbor EGFR exon 19 del with EFIRM were confirmed by cobas assay. Furthermore, these three patients with EGFR L858R mutations also perfectly matched the mutation results for saliva, plasma, and tissue samples. The other 11 patients were EGFR wild-type in both saliva and tissue samples, which meant that there was a single false positive L858R mutation in plasma sample detection by EFIRM. The specificity and sensitivity was 90.9% and 100%, respectively, by plasma. In addition, all of the patients with EGFR mutation were histologically diagnosed with adenocarcinoma (3 stage III, 2 stage II, and 1 stage I), which indicated that EFIRM assay could maintain a high sensitivity in different cancer stages.

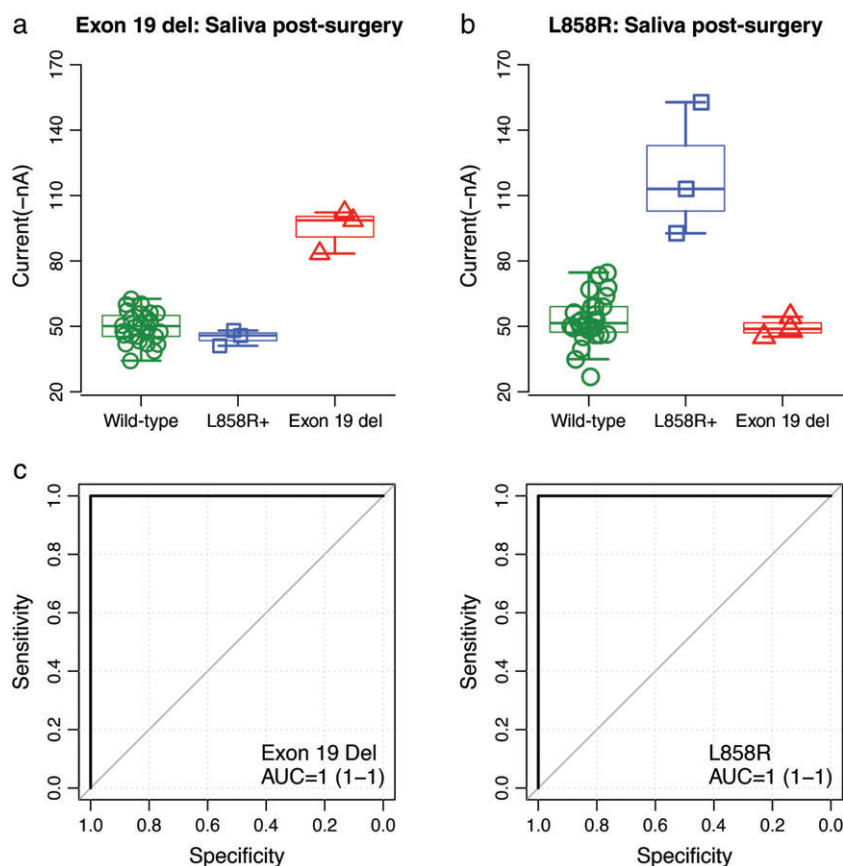


Figure 2 Blinded and randomized detection of epidermal growth factor receptor (EGFR) mutations by electric field-induced release and measurement assay in saliva collected after surgery from patients with non-small cell lung cancer: (a) the probe for EGFR exon 19 deletion in saliva; (b) the probe for L858R in saliva; and (c) receiver operating characteristic curves for detecting EGFR exon 19 deletion (area under the curve [AUC]= 1) and L858R mutations (AUC = 1), respectively.

The correlation of EGFR mutation detected before and after surgery

We compared the results from the saliva and plasma collected before and after surgery to evaluate whether surgery affected EGFR mutation status. The strength of the amperometric currents in the saliva samples collected before surgery correlated with those after surgery using the L858R and exon 19 probes (Table 2, $R = 0.86$ in exon 19 del; $R = 0.98$ in L858R). Similar results were obtained in plasma samples (Table 2, $R = 0.73$ in exon 19 del; $R = 0.94$ in L858R). Additionally, regardless of whether samples were collected before or after surgery, the amperometric currents between saliva and plasma had a strong correlation (Table 2, before surgery: $R = 0.87$ in exon 19 del and $R = 0.67$ in L858R; after surgery: $R = 0.79$ in exon 19 del and $R = 0.70$ in L858R).

Discussion

Liquid biopsy to detect actionable mutations in NSCLC in clinical practice is primarily based on digital droplet PCR (ddPCR) and/or next generation sequencing technologies with performance at ~70–80% concordance with tissue-based genotyping. Technologies that will permit 100% concordance detection (sensitivity) will provide the ultimate complementation to the tumor-specific fingerprint (specificity) for unambiguous detection of the tumor in a non-invasive setting.^{3–14} To our knowledge, this is the first pilot study aimed at evaluating a novel saliva-based EGFR mutation detection system for lung cancer in Mainland China. Our findings clearly show that saliva and plasma assays conducted using the EFIRM method have high concordance with biopsy tissue-based testing for lung cancer EGFR mutation status. EFIRM liquid biopsy is accurate, non-

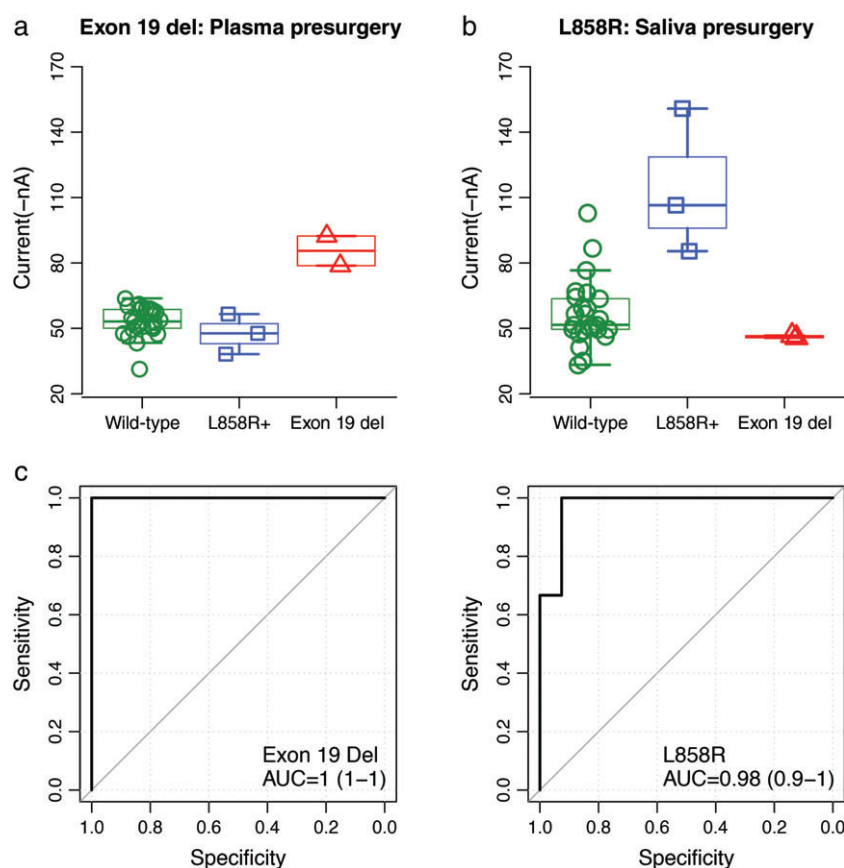


Figure 3 Blinded and randomized detection of epidermal growth factor receptor (EGFR) mutations by electric field-induced release and measurement assay in plasma collected before surgery from patients with non-small cell lung cancer: (a) the probe for EGFR exon 19 deletion; (b) the probe for L858R; and (c) receiver operating characteristic curves for detecting EGFR exon 19 deletion (area under the curve [AUC]= 1) and L858R mutations (AUC = 0.98), respectively.

invasive, and rapid. It can be used in continuous monitoring EGFR mutation status during EGFR-TKI treatment.

We demonstrated that the amperometric currents of the EFIRM signals from saliva were highly correlated with those from plasma using the probe designed for p.E746-A750del and p.L858R, regardless of whether the sample was collected presurgery or post-surgery (0.70s range). These results suggest that the amount of EGFR mutant DNA in saliva and plasma is correlated. This may have a mechanistic implication, suggesting a link between the peripheral circulatory system and the salivary glands. Previous studies of this link have also found similarly strong correlations.²² However, the precise mechanism by which lung cancer cells release the EGFR mutant DNA and disperse it via the blood to distal sites remains to be established.

Another interesting finding in our study was the strong correlations found between presurgery and post-surgery saliva assays (0.86 and 0.98) and plasma (0.73 and 0.94).

This data implies that surgery may not influence EGFR mutant DNA status in saliva or plasma. Saliva collected after biopsy may result in tumor cells or cell-free DNA that make their way into the saliva after tissue disruption, demonstrating that the detection of EGFR mutation in saliva was not a result of biopsy materials leaking into saliva.

Although this cohort of tests was only conducted on a small scale, there were still overlapping current values of EGFR wild type and mutation type at exon 21 L858R in plasma, which may have led to false-positive results. Previous studies have demonstrated a similar phenomenon at L858R with an unclear threshold between wild type and mutation type.²² A large prospective study will be conducted to determine the adequate and optimal estimate of a threshold to decide the balance between false positive and false negative rates.

Electric field-induced release and measurement can detect EGFR mutations in liquid biopsy from NSCLC

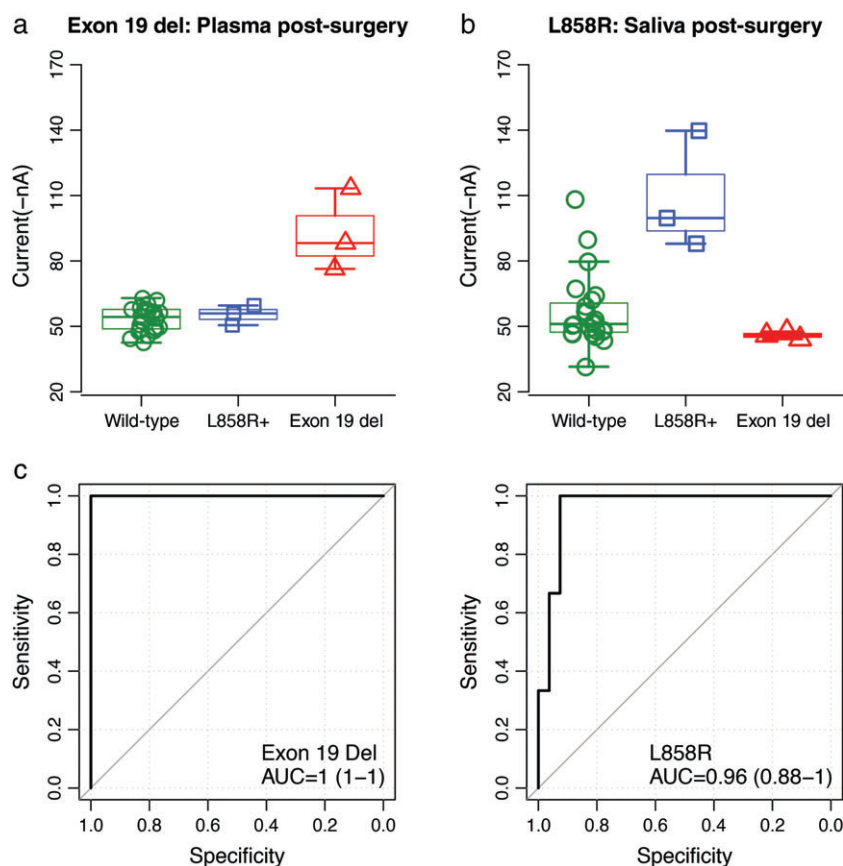


Figure 4 Blinded and randomized detection of epidermal growth factor receptor (EGFR) mutations by electric field-induced release and measurement assay in plasma collected after surgery from patients with non-small cell lung cancer: (a) the probe for EGFR exon 19 deletion; (b) the probe for L858R; and (c) receiver operating characteristic curves for detecting EGFR exon 19 deletion (area under the curve [AUC = 1] and L858R mutations (AUC = 0.96), respectively.

patients, but at present this work has mainly focused on EGFR L858R and exon 19 del mutations. Because the second T790M EGFR mutation accounts for half of the TKI-

resistant cases, monitoring the T790M mutation would be useful to estimate EGFR-TKI resistance. Therefore, development of a new probe designed for EFIRM based on T790M mutation is necessary, and this approach, as with other liquid biopsy-based EGFR mutation assays (such as circulating tumor cells or cell-free circulating tumor DNA), might be a valid noninvasive alternative to biopsy, as well as a better tailoring of individualized precision therapy by monitoring the response to treatment in NSCLC patients.

In conclusion, our results provide evidence that EFIRM detection of EGFR mutation in the saliva of patients with lung cancer is accurate, non-invasive, and rapid.

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Table 2 The correlation of EGFR mutation detected before and after surgery or biopsy

	Probe type	Pearson
Saliva: pre/post surgery	Exon 19 del	0.86
	L858	0.98
Plasma: pre/post surgery	Exon 19 del	0.73
	L858	0.94
Pre surgery: saliva/plasma	Exon 19 del	0.87
	L858	0.67
Post surgery: saliva/plasma	Exon 19 del	0.79
	L858	0.70

EGFR, epidermal growth factor receptor.

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Disclosure

David Wong is a co-founder of RNameTRIX Inc., a molecular diagnostic company. He holds equity in RNameTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNameTRIX. Intellectual property invented by David Wong and patented by the University of California has been licensed to RNameTRIX.

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